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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)* Patents ADP number *(if you know it)* 810127 1001 If the applicant is a corporate body, give the country/state of its incorporation	GlaxoSmithKline Biologicals s.a. Rue de l'Institut 89, B-1330 Rixensart, , Belgium Belgian
4.	Title of the invention	Immunogenic Composition
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* Patents ADP number *(if you know it)* 8072555006	Corporate Intellectual Property GlaxoSmithKline Corporate Intellectual Property (CN9 25.1) 980 Great West Road BRENTFORD Middlesex TW8 9GS
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Immunogenic composition

The present invention relates to immunogenic compositions comprising a dried solid formulation of IPV which retains immunogenicity, preferably further comprising a bacterial polysaccharide. The invention also includes a vaccine comprising a dried solid formulation of IPV and a bacterial polysaccharide. A further aspect of the invention is a novel process for preserving inactivated polio virus (IPV) as a dried solid. This process comprises preparing a sample by suspending or dissolving IPV and a bacterial polysaccharide in a solution of a stabilising agent and subjecting the sample to temperature and pressure conditions which result in solvent being lost from the sample. Pressure and temperature conditions may be maintained or adjusted so that solvent is removed and the sample dries to form a solid. Such formulations may be reconstituted prior to use or used directly.

IPV is well known as a component of vaccines, however, it is formulated as a liquid, for example in Infanrix penta ®. The process of freeze-drying IPV has been associated with the loss of antigenicity so that it is difficult to formulate an effective vaccine comprising a solid form of IPV. Solid vaccine formulations are known, particularly in the case of bacterial polysaccharides. The PRP polysaccharide of *Haemophilus influenzae* b (Hib) is frequently formulated as a dried solid, for example in Infanrix hexa ® (WO99/48525).

There are several reasons why a dried formulation of IPV would be advantageous. Dried formulations have good storage properties and may be useful in increasing the shelf life of a vaccine containing IPV. The possibility of drying IPV also makes IPV a more flexible vaccine constituent and enables it to be formulated in new combinations which were not previously possible. Some vaccines contain liquid and dried solid components which are mixed just prior to administration (for example Infanrix hexa ®). Infanrix Hexa contains a dried Hib component which is reconstituted with DTPa-HepB-IPV just prior to use. By formulating IPV together with Hib as a dried solid, it would be possible to add further components to the liquid part of the vaccine, which might otherwise be incompatible with IPV.

Several techniques for drying vaccine components are known in the art. Traditionally, this has been accomplished using the process of freeze drying in which a solution of the substance is made and the sample is frozen. During the primary drying phase, most of the water is removed by sublimation from ice under reduced pressure conditions and a porous 'cake' is formed. This is usually followed by a secondary drying phase when the pressure and temperature are changed and water is evaporated from the solid 'cake'. The resulting lyophilised sample has improved stability compared to a liquid formulation. However, the freeze drying process is lengthy and can be the rate limiting step in a production process.

Product variability is also a problem when many samples are being batch lyophilised in a large dryer unit. The conditions on the shelves of the freeze dryer vary between different positions leading to samples lyophilising at different rates under different conditions. For certain biological materials such as live virus, there can be significant loss of activity during the freeze drying process (Pikal (1994) ACS Symposium 567: 120-133). Many freeze dried substances are still unstable at ambient temperature (Carpenter et al (1994) ACS Symposium 567; 134-147).

Damage caused by the process of freezing may be circumvented to some degree by the use of cryoprotectants such as polyols. Further improvements on the process of lyophilisation have also been made by avoiding freezing the sample during the process and removing water by boiling (WO96/40077; US6306345). This method involves preparing a mixture of a glass-matrix forming material in a suitable solvent together with the sample to be preserved, evaporating bulk solvent from the mixture to obtain a syrup, exposing the syrup to a pressure and temperature sufficient to cause boiling of the syrup and removing residual solvent.

A similar method was described in US5,766,520, in which the process involves partially removing the water to form a viscous fluid and further subjecting the syrup to vacuum to cause it to 'boil' and further drying at temperatures substantially lower than 100 °C. This method still suffers from some of the problems of conventional

freeze-drying. When the process is carried out in a large freeze-dryer, samples will dry at different rates depending on their position on the shelf and this leads to different samples loosing different amount of activity during the drying process. This leads to a lack of consistency within a batch.

To date, no successful example of making a dried solid vaccine formulation of IPV that retains a high degree of antigenicity has been reported.

The present invention discloses a dried solid form of IPV which retains a high degree of antigenicity. The presence of a stabilising agent is crucial to the preservation of antigens and polyols are shown to be effective. IPV is preferably dried in the presence of a bacterial polysaccharide which leads to preservation of a higher percentage of the original antigens. The present invention encompasses methods of preserving a composition comprising IPV and a bacterial polysaccharide, wherein the antigenic integrity of the labile IPV is maintained. Lyophilisation of IPV in the presence of polysaccharides leads to an improvement in antigen retention for IPV compared to lyophilisation of IPV alone. In addition, the immunogenicity of Hib is also enhanced by being formulated together with IPV as a dried solid. In particular, when reconstituted extemporaneously with liquid DTP vaccines (described below), the inventors have found that Hib titres are not as reduced by the aluminium hydroxide component of the DTP vaccine as would have been the case without the presence of IPV.

The method of drying used can also influence the antigen retention of IPV. A foam drying process for drying IPV and a bacterial polysaccharide was more effective at retaining antigens of IPV than conventional freeze drying techniques. Surprisingly, the inclusion of a freezing step in the foam drying process did not lead to antigen destruction but rather led to the development of a quick and effective preservation process.

The invention provides a dried formulation of IPV which will have benefits of storage stability. The lyophilised cake can be reconstituted quickly and easily just prior to

administration. Where the preferred foam drying process is used, the foamed cake is particularly easily reconstituted due to the greater surface area of the cake.

Additional benefits of a dried solid formulation of IPV and Hib include enhanced immunogenicity of the Hib component. It is well known that in multi-component vaccines, other parts of the vaccine formulation can lead to interference with Hib immunogenicity (WO96/40242, WO97/00697). The inclusion of IPV in a dried formulation with Hib can reduce this problem, especially if the dried IPV-Hib composition is mixed with diphtheria, tetanus and pertussis conponents prior to administration.

Although lyophilisation of IPV in the presence of a bacterial polysaccharide is possible using a conventional freeze drying approach, it is preferred to use a foam drying technique. This process results in even greater antigen retention in IPV and the resultant cake is also easier and quicker to reconstitute. The process also has advantages in being quicker and more energy efficient than standard freeze-drying techniques. Since the lyophilisation step is often the rate limiting step in vaccine production, the use of the preferred process would result in higher levels of vaccine production without additional investment in plant. The introduction of a freezing step into the preferred process also leads to improved batch reproducibility.

Description of figures

Figure 1 – Photographs of vials containing the preservation sample at different stages of the foam drying process.

A - Shows the appearance of the preservation samples as inserted into the freeze drying as a liquid formulation.

B - Shows the appearance of the preservation samples as the pressure is reduced to 1.5mbars. The samples begin to freeze at slightly different rates due to differing conditions in each vial.

C - Shows the appearance of the preservation samples at 0.1mbars, where all samples have become completely frozen.

D - Shows the appearance of the preservation samples as the pressure is increased to 0.8 – 3.5mbars. A foamed glass is formed as the preservation sample foams and solvent evaporates.

Detailed description

Immunogenic compositions of the invention

The invention includes immunogenic compositions, formulated as a dried solid comprising IPV and a stabilising agent, in which the antigenicity of IPV is retained following reconstitution. The dried solid formulation of IPV is capable of generating an immune response, preferably an effective immune response, against polio virus, preferably after reconstitution and inoculation.

IPV is defined as inactivated polio virus (preferably comprising types 1, 2 and 3 as is standard in the vaccine art, most preferably the Salk polio vaccine). A vaccine dose of IPV contains 20-80, preferably 40 or 80 D-antigen units of type 1 (Mahoney), 4-16, preferably 8 or 16 D-antigen units of type 2 (MEF-1) and 20-64, preferably 32 or 64 D-antigen units of type 3 (Saukett).

A dried solid formulation is a formulation which has had solvent removed by a process of lyophilisation or desiccation so that less than 5%, 4%, preferably 3%, 2% or most preferably 1% solvent remains. The term 'dried solid' comprises glasses, rubbers or crystalline solids with a solid appearance. Any of the methods described above can be used to make such a dried solid. Solvent is removed by sublimation, boiling or evaporation, preferably by evaporation. Preferably, the drying process utilised retains the IPV antigens so that an ELISA on the reconstituted dried solid, using antibodies against polio virus type 1, 2 and/or 3, gives results that are over 40%, 50%, preferably 60%, 70%, more preferably 80% or 90% the level achieved using the undried IPV. In vivo experiments in which the dried formulation, after reconstitution is inoculated into an animal, preferably a mouse may also be used to assess the degree of antigen retention.

Immunogenic compositions of the invention are formulated as a dried solid comprising IPV and preferably a bacterial polysaccharide. The bacterial polysaccharide comprises capsular polysaccharides derived from any bacterium, preferably one or more of *Neisseria meningitidis*, *Haemophilus influenzae* b, *Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* or *Staphylococcus epidermidis*.

Preferably the PRP capsular polysaccharide of *Haemophilus influenzae* b is present as a dried solid. In a further preferred embodiment, the immunogenic composition comprises dried solid formulations of capsular polysaccharides derived from one or more of serogroups A, C, W-135 and Y of *Neisseria meningitidis* (meningococcal polysaccharides). A further preferred embodiment comprises dried solid formulations of capsular polysaccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). A further preferred embodiment contains the Type 5, Type 8 or 336 capsular polysaccharides of *Staphylococcus aureus*. A further preferred embodiment contains the Type I, Type II or Type III capsular polysaccharides of *Staphylococcus epidermidis*. A further preferred embodiment contains the Type Ia, Type Ic, Type II or Type III capsular polysaccharides of Group B streptocoocus. A further preferred embodiment contains the capsular polysaccharides of Group A streptococcus, preferably further comprising at least one M protein and more preferably multiple types of M protein.

In one embodiment of the invention, the bacterial polysaccharides are full length, being purified native polysaccharides. In an alternative embodiment of the invention, the polysaccharides are sized between 2 and 20 times, preferably 2-5 times, 5-10 times, 10-15 times or 15-20 times, so that the polysaccharides are smaller in size for greater manageability. Oligosaccharides are used in a preferred embodiment. Oligosaccharides typically contain between 2 and 20 repeat units.

The invention further includes immunogenic compositions comprising more than one bacterial polysaccharide and IPV as a dried solid. Preferably, IPV is combined with one or more of Hib (*Haemophilus influenzae* type b) PRP polysaccharide and/or meningococcal A, C, W and/or Y polysaccharides and/or pneumococcal polysaccharides. Most preferably the active agents comprise, IPV and Hib; IPV and MenC; IPV, Hib and MenC; Hib and MenC; IPV and MenA and C; Hib and Men A and C; IPV, Hib, Men A and C; Hib, Men C and Y; or IPV, Hib, Men C and Y.

The above particularised active agents may also comprise one or more pneumococcal capsular polysaccharides as described below.

In the above compositions where polysaccharides are used, oligosaccharides may also be employed (as defined below).

Although these compositions may be adjuvanted (as described below), they are preferably unadjuvanted or preferably do not comprise aluminium salts.

Preferably the polysaccharides or alogosaccharides are conjugated to a peptide or carrier protein comprising T-helper epitopes (as described below).

Capsular polysaccharides present in immunogenic compositions of the invention are unconjugated or conjugated to a carrier protein such as tetanus toxoid, tetanus toxoid fragment C, diphtheria toxoid, CRM197, pneumolysin, Protein D (US6342224). Tetanus toxin, diphtheria toxin and pneumolysin are detoxified either by genetic mutation and/or preferably by chemical treatment. A preferred embodiment of the invention has Hib conjugated to tetanus toxoid.

Where more than one conjugated polysaccharide is present in the immunogenic composition of the invention, the polysaccharides are conjugated to the same carrier protein or to different carrier proteins. Preferred embodiments of the invention contain meningococcal polysaccharides conjugated to a carrier protein. Where conjugated

Hib and meningococcal polysaccharides are present, they are conjugated to the same carrier protein or to different carrier proteins.

The polysaccharide conjugate may be prepared by any known coupling technique. In a preferred coupling technique, the polysaccharide is coupled via a thioether linkage. This conjugation method relies on activation of the polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may thus be coupled directly or via a spacer group to an amino group on the carrier protein. Preferably, the cyanate ester is coupled with hexane diamine and the amino-derivatised polysaccharide is conjugated to the carrier protein using heteroligation chemistry involving the formation of the thioether linkage. Such conjugates are described in PCT published application WO93/15760 Uniformed Services University.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide activated polysaccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256).

Polysaccharides which are incorporated as part of the immunogenic composition of the invention may be unabsorbed or absorbed onto an adjuvant , preferably an aluminium salt (aluminium phosphate or aluminium hydroxide), most preferably aluminium phosphate.

Immunogenic compositions of the invention comprise a stabilising agent which can help to prevent damage during the desiccation process. Any of the stabilising agent described below, including glass forming polyols can be incorporated into the immunogenic composition, whether as a freeze dried or a foamed glass composition

using the processes of the invention. Preferred stabilising agents include sucrose, sorbitol, lactose and trehalose.

The preferred combinations, dried by the process of the invention may be combined with other antigens in a combination vaccine which are desiccated or liquid formulations which are used to reconstitute the dried components.

Additional components

Dried solid formulations of the invention incorporating IPV and a bacterial polysaccharide may additionally be formulated with other vaccine components. A preferred vaccine contains a dried solid formulation of IPV and a bacterial polysaccharide which may be mixed with a liquid formulation comprising additional vaccine components. After reconstitution of the solid components with the liquid components, the complete vaccine is administered by injection.

The additional components include capsular polysaccharides derived from one or more of *Neisseria meningitidis, Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* or *Staphylococcus epidermidis*. In a preferred embodiment, the immunogenic composition comprises capsular polysaccharides derived from one or more of serogroups A, C, W-135 and Y of *Neisseria meningitidis*. A further preferred embodiment comprises capsular polysaccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F).. A further preferred embodiment contains the Type 5, Type 8 or 336 capsular polysaccharides of *Staphylococcus aureus*. A further preferred embodiment contains the Type I, Type II or Type III capsular polysaccharides of *Staphylococcus epidermidis*. A further preferred embodiment contains the Type Ia, Type Ic, Type II or Type III capsular polysaccharides of Group B streptocoocus. A further preferred embodiment would

contain the capsular polysaccharides of Group A streptococcus, preferably further comprising at least one M protein and more preferably multiple types of M protein.

The immunogenic composition of the invention may be formulated with protein antigens. Preferred pneumococcal proteins antigens are those pneumococcal proteins which are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal tranducer, or lipoprotein of *Streptococcus pneumoniae*, or fragments thereof. Particularly preferred proteins include, but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC and transmembrane deletion variants thereof (WO 97/09994 - Briles et al); PsaA and transmembrane deletion variants thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); pneumococcal choline binding proteins and transmembrane deletion variants thereof; CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP70 (WO 96/40928); PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14); M like protein, (EP 0837130) and adhesin 18627, (EP 0834568). Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390.

Preferred Neisserial proteins to be formulated with the immunogenic composition of the invention include TbpA (WO93/06861; EP586266; WO92/03467; US5912336),

TbpB (WO93/06861; EP586266), Hsf (WO99/31132), NspA (WO96/29412), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (also known as D15) (WO00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO96/31618 see SEQ ID NO:38), FrpA or FrpC or a conserved portion in commen to both of at least 30, 50, 100, 500, 750 amino acids (WO92/01460), LbpA and/or LbpB (PCT/EP98/05117; Schryvers et al Med. Microbiol. 1999 32: 1117), FhaB (WO98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), MltA (WO99/57280) and ctrA (PCT/EP00/00135).

The immunogenic composition is preferably formulated with antigens providing protection against one or more of Diphtheria, tetanus and *Bordetella pertussis* infections. The pertussis component may be killed whole cell *B. pertussis* (Pw) or is preferably acellular pertussis (Pa) which contains at least one antigen (preferably two or all three) from PT, FHA and 69kDa pertactin. Typically, the antigens providing protection against Diphtheria and tetanus are Diphtheria toxoid and tetanus toxoid. The toxoids are chemically inactivated toxins or toxins inactivated by the introduction of point mutations.

Alternatively the foamed glass of the invention may be provided as a kit with the foamed glass in one container and liquid DTPa or DTPw in another container. The foamed glass is reconstituted with the liquid DTPa or DTPw vaccine (preferably extemporaneously) and administered as a single vaccine. The DTPa or DTPw vaccine typically is adjuvanted at least in part with aluminium hydroxide (for instance Infanrix ® and Tritanrix ® vaccines of GlaxoSmithKline Biologicals s.a.).

The immunogenic composition is optionally formulated with one or more antigens that can protect a host against non-typeable *Haemophilus influenzae*, RSV and/or one or more antigens that can protect a host against influenza virus. Preferred non-typeable *H. influenzae* protein antigens include Fimbrin protein (US 5766608) and fusions comprising peptides therefrom (eg LB1 Fusion) (US 5843464 - Ohio State Research Foundation), OMP26, P6, protein D, TbpA, TbpB, Hia, Hmw1, Hmw2, Hap, and D15.

Preferred influenza virus antigens include whole, live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof.

Preferred RSV (Respiratory Syncytial Virus) antigens include the F glycoprotein, the G glycoprotein, the HN protein, the M protein or derivatives thereof.

Combination vaccines comprising DTPw-IPV-Hib or DTPa-IPV-Hib are known in the art. However there are problems associated with combinations such as these, containing Hib and other vaccine components. The antibody titres raised against the Hib component are frequently lower than those elicited by the same dose of Hib inoculated separately, due to interference with other components of the vaccine such as aluminium hydroxide adjuvant. The immunogenic composition of the invention provides a solution to this problem.

The immunogenic compositions of the invention may form part of a vaccine kit in which IPV and Hib are present in one component of the kit and further components, as described above, are present in a second component. The two components are mixed together just before administration of the vaccine. In such formulations, the component comprising IPV and Hib is preferably a dried solid, although it is optionally formulated as a liquid. This sort of formulation results in a solution to the Hib interference problem with antibody titres against the Hib component being at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 500% higher than those elicited by the same dose of Hib in a combination vaccine where IPV has been omitted from the formulation or where IPV and Hib have not been included in the same component of the vaccine kit.

Vaccines of the invention

The immunogenic compositions of the invention described above are preferably formulated as a vaccine. Preferably, the vaccine contains an amount of an adjuvant sufficient to enhance the immune response to the immunogen. Suitable adjuvants include, but are not limited to, aluminium salts such as aluminium hydroxide and aluminium phosphate, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, non-ionic block copolymer surfactants, Quil A, cholera toxin B subunit, polphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) Nature 344:873-875. For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant can be used.

The vaccine formulations of the invention are preferably reconstituted prior to use. Reconstitution involves the mixing of a liquid component of the vaccine with the dried solid formulation of the invention. The invention also encompasses a container with a water repellent internal surface containing the immunogenic composition or vaccine of the invention.

As with all immunogenic compositions or vaccines, the immunologically effective amounts of the immunogens must be determined empirically. Factors to be considered include the immunogenicity, whether or not the immunogen will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier, route of adminstrations and the number of immunising dosages to be adminstered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The substance can be present in varying concentrations in the immunogenic composition of the invention. Typically, the minimum concentration of the substance is an amount necessary to achieve its intended use, while the maximum concentration is the maximum amount that will remain in solution or homogeneously suspended within the initial mixture. For instance, the minimum amount of a therapeutic agent is preferably one which will provide a single therapeutically effective dosage. Super-

saturated solutions can also be used if the foamed glass is formed prior to crystallisation. For bioactive substances, the minimum concentration is an amount necessary for bioactivity upon reconstitution and the maximum concentration is at the point at which a homogeneous suspension cannot be maintained. In the case of single-dosed units, the amount is that of a single therapeutic application Generally, it is expected that each dose will comprise 1-100ug of protein antigen, preferably 5-50ug and most preferably 5-25ug. Preferred doses of bacterial polysaccharides are 10-20ug, 10-5ug, 5-2.5ug or 2.5-1ug. The preferred amount of the substance varies from substance to substance but is easily determinable by one of skill in the art.

Methods of the invention

The methods of the invention are capable of preserving a composition comprising IPV, resulting in a composition of IPV in which antigenicity is retained. Preferably, a bacterial polysaccharide and/or a stabilising agent is incorporated in the sample to be dried. A method of the invention involves freeze drying IPV and comprises the steps of:

- preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent; preferably a bacterial polysaccharide and/or a glass forming polyol are present in the preservation sample;
- subjecting the preservation sample to such temperature and pressure conditions that solvent is lost from the preservation sample ; and
- removing solvent until the preservation sample dries to form a solid.

A further method of the invention involves foam drying, comprising the steps of:

- preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent; preferably a bacterial polysaccharide and/or a glass forming polyol are present in the preservation sample;
- subjecting the preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and
- removing solvent until the foam dries to form a solid.

A preferred foam drying method of the invention uses a container with a water repellent interior surface and contains the steps of:

- preparing a preservation sample by suspending or dissolving IPV and preferably a bacterial polysaccharide in a solution of a stabilising agent;
- inserting the preservation sample into a container with a water repellent interior surface;
- subjecting the container containing the preservation sample to such temperature and pressure conditions so that the preservation sample forms a foam;
- removing solvent until the foam dries to form a solid.

The foam drying methods of the invention described above optionally comprise a freezing step. The preservation sample may be wholly or partially frozen. Therefore some methods of the invention comprise the steps of:

- preparing an at least partially frozen preservation sample by suspending or dissolving IPV and preferably a bacterial polysaccharide in a solution of a stabilising agent and freezing the mixture;
- subjecting the at least partially frozen preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and
- removing solvent until the foam dries to form a solid.

The freezing step of the above method is preferably by the process of quench freezing in which reduction of pressure is the cause of freezing by evaporation. This causes rapid freezing of the sample which leads to less antigen loss. Therefore a process of the invention includes the steps of:

- preparing a preservation sample by suspending or dissolving IPV and preferably a bacterial polysaccharide in a solution of a stabilising agent;
- subjecting the preservation sample to reduced pressure such that the preservation sample becomes at least partially frozen;
- subjecting the at least partially frozen preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and
- removing solvent until the foam dries to form a solid.

Stabilising agent

The stabilising agent to be used in the methods of the invention will preferably be glass forming polyols. Suitable materials include, but are not limited to, all polyols, including carbohydrate and non-carbohydrate polyols. Preferably the stabilising polyol enables the active agent to be stored without substantial loss of activity by denaturation, aggregation or other means. Particularly suitable materials include sugars, sugar alcohols and carbohydrate derivatives. Preferably, the glass forming polyol is a carbohydrate or derivatives thereof, including glucose, maltulose, iso-maltulose, lactulose, sucrose, maltose, lactose, iso-maltose, maltitol, lactitol, palatinit, trehalose, raffinose, stachyose, melezitose or dextran, most preferably trehalose, sucrose, sorbitol, raffinose, mannitol, lactose, lactitol or palatinit.

Bacterial polysaccharides act as a stabilising agent and preferred embodiments of the invention incorporate bacterial polysaccharides playing a dual role of stabilising agent and immunogen.

Carbohydrates include, but are not limited to, monosaccharides, disaccharides, trisaccharides, oligosaccharides and their corresponding sugar alcohols, polyhydroxyl compounds such as carbohydrate derivatives and chemically modified carbohydrates, hydroxyethyl starch and sugar copolymers. Both natural and synthetic carbohydrates are suitable for use. Synthetic carbohydrates include, but are not limited to, those which have the glycosidic bond replaced by a thiol or carbon bond. Both D and L forms of the carbohydrates may be used. The carbohydrate may be non-reducing or reducing. Where a reducing carbohydrate is used, the addition of inhibitors of the Maillard reaction is preferred.

Reducing carbohydrates suitable for use in the invention are those known in the art and include, but are not limited to, glucose, maltose, lactose, fructose, galactoase, mannose, maltulose and lactulose. Non-reducing carbohydrates include, but are not limited to, non-reducing glycosides of polyhydroxyl compounds selected from sugar alcohols and other straight chain polyalcohols. Other useful carbohydrates include

raffinose, stachyose, melezitose, dextran, sucrose, cellibiose, mannobiose and sugar alcohols. The sugar alcohol glycosides are preferably monoglycosides, in particular the compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose.

Particularly preferred carbohydrates are trehalose, sucrose, sorbitol, maltitol, lactitol, palatinit and glucopyranosyl-1→6–mannitol.

Amino acids can act as stabilising agents and can be used by themselves and preferably in combination with a polyol. Preferred amino acids include glycine, alanine, arginine, lysine and glutamine although any amino acid, or a combination of amino acids, peptide, hydrolysed proteins or protein such as serum albumin can act as a stabilising agent.

The concentration of the stabilising agent used in the process of the invention may be between 1% and 50% weight/volume, preferably 1-5%, 5-10%, 5-10%, 15-20%, 20-25% or 25-50%, most preferably less than 25%. The amounts of stabilising agent required is proportional to the amount of salts present. Therefore, although levels of stabilising agent between 3% and 10% are preferred, higher concentrations of 10% to 25% may be required to dry samples with a high salt content.

Container

Different mixtures and various container shapes and sizes can be processed simultaneously. Ideally, the container size used is sufficient to contain the initial mixture and accommodate the volume of the foamed glass formed thereof. Typically, this is determined by the mass of the glass forming material, the surface area of the container and the conditions of the foamed glass formation. The mass of glass forming material must be sufficient to give viscous syrup to be foamed which translates practically as a minimal mass per unit area of container surface. This ratio varies from mixture to mixture and container used, but is easily determined

empirically by one skilled in the art by following the procedures set forth herein. Any such containers can be used, including Wheaton moulded and tube-cut vials.

The process of the invention preferably uses containers with a water repellent interior surface. This is achieved through coating the interior surface with a hydrophobic composition, for instance by siliconisation. Siliconisation is achieved by processes that are well known to those skilled in the art. In one method, the container is siliconised by rising the interior of the container with an emulsion of silicone, followed by processing through an oven at high temperature, typically 350 °C. Alternatively, the water repellent interior surface is achieved by the container being made of a water repellent composition.

The water repellent interior surface of the container makes foam formation more likely to occur and more reproducible. This allows lower polyol concentrations to be used in the preservation sample which in turn decreases the length of time necessary to dry the sample, reduces the effect of Maillard reactions or other interactions with the polyol harming the active agent. Where the preservation samples comprises a vaccine, the resultant foamed glass is reconstituted quickly and easily due to the lower amount of polyol present and the resultant vaccine solution is less viscous, allowing easier administration.

Although singular forms may be used herein, more than one stabilising agent, more than one additive, and more than one substance may be present. Effective amounts of these components are easily determined by one skilled in the art.

<u>Solvent</u>

The preservation sample is made by dissolving/suspending IPV and a stabilising agent in water to make an aqueous solution. Preferably, water is present in the preservation sample at a level of 5 to 98% by volume, more preferably 80-98% by volume, most preferably 85-98% by volume.

The volume of solvent can vary and will depend upon the stabilising agent and the substance to be incorporated as well as any additives. The minimum volume required is an amount necessary to solubilise the various components. However, homogeneously dispersed suspensions of the substance(s) can also be used. Suitable amounts of the components in specific embodiments are easily determinable by those skilled in the art in light of the examples provided herein.

Various additives can be put into the preservation sample. Typically, the additives enhance foam formation and /or the drying process or contribute to the solubilization of the substance. Alternatively, the additives contribute to the stability of the substance incorporated within the solid. One or more additives may be present.

As an example, addition of volatile/effervescent salts allows larger initial volumes and results in higher surface area within the foamed glass, thus effecting superior foam formation and more rapid drying. As used herein, volatile salts are salts which volatilise under the conditions used to produce a foamed glass. Examples of suitable volatile salts include, but are not limited to, ammonium acetate, ammonium bicarbonate and ammonium carbonate. Salts that decompose to give gaseous products also effect enhanced foam formation and more rapid drying. Examples of such salts are sodium bicarbonate and sodium metabisulphite. Preferably, the volatile salts are present in an amount of from about 0.01 to 5 M. Concentrations of up to 5 M are suitable for use herein. The resultant foamed glass has uniform foam conformation and is significantly drier compared to foamed glass in which volatile/effervescent salts are not used.

Another suitable additive is a foam stabilising agent, which can be used in combination with either the volatile or decomposing salt. This may either be a surface active component such as an amphipathic molecule (i.e. such as phospholipids and surfactants) or an agent to increase the viscosity of the foaming syrup, such as a thickening agent such as guar gum and their derivatives.

Another additive is an inhibitor of the Maillard reaction. Preferably, if the substance and/or glass matrix-forming material contains carbonyl and amino, imino or guanidino groups, the compositions further contain at least one physiologically acceptable inhibitor of the Maillard reaction in an amount effective to substantially prevent condensation of amino groups and reactive carbonyl groups in the composition. The inhibitor of the Maillard reaction can be any known in the art. The inhibitor is present in an amount sufficient to prevent, or substantially prevent, condensation of amino groups and reactive carbonyl groups. Typically, the amino groups are present on the substance and the carbonyl groups are present on the glass matrix forming material, or the converse. However, the amino acids and carbonyl groups may be intramolecular within either the substance or the carbohydrate.

Various classes of compounds are known to exhibit an inhibiting effect on the Maillard reaction and hence to be of use in the compositions described herein. These compounds are generally either competitive or non-competitive inhibitors of the Maillard reaction. Competitive inhibitors include, but are not limited to, amino acid residues (both D and L), combinations of amino acid residues and peptides. Particularly preferred are lysine, arginine, histidine and tryptophan. Lysine and aarginine are the most effective. There are many known non-competitive inhibitors. These include, but are not limited to, aminoguanidine and derivatives and amphotericin B. EP-A-0 433 679 also describes suitable Maillard inhibitors which include 4-hydroxy-5, 8-dioxoquinoline derivatives.

Active agents

The methods of the invention are used to preserve inactivated polio virus (IPV – preferably comprising types 1, 2 and 3 as is standard in the vaccine art, most preferably the Salk polio vaccine). IPV contains 20-80, preferably 40 or 8- D-antigen units of type 1 (Mahoney), 4-20, preferably 8 or 16 D-antigen units of type 2 (MEF-1) and 20-64, preferably 32 or 64 D-antigen units of type 3 (Saukett). The IPV vaccine formulation is suitable for injection after reconstitution in an aqueous solution which preferably contains additional vaccine components.

The bacterial polysaccharide incorporated by the process of the invention are for example capsular polysaccharides derived from one or more of *Neisseria meningitidis*, *Haemophilus influenzae* b, *Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* or *Staphylococcus epidermidis*, preferably the PRP capsular polysaccharides of *Haemophilus influenzae*.. Preferred capsular polysaccharides also include those derived from one or more of serogroups A, C, W-135 and Y of *Neisseria meningitidis*. Further preferred capsular polysaccharides are derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). A further preferred embodiment contains the Type 5, Type 8 or 336 capsular polysaccharides of *Staphylococcus aureus*. Further preferred polysaccharides include the Type I, Type II or Type III capsular polysaccharides of *Staphylococcus epidermidis*, the Type Ia, Type Ic, Type II or Type III capsular polysaccharides of Group B streptocoocus. Further preferred polysaccharides include the capsular polysaccharides of Group A streptococcus, preferably further comprising at least one M protein and more preferably multiple types of M protein.

Preferred combinations of active agents to be preserved using the process of the invention comprise IPV. Preferably, IPV is combined with bacterial polysaccharides comprising one or more of Hib PRP polysaccharide and/or meningococcal A, C, W and/or Y polysaccharides and/or pneumococcal polysaccharides. Preferred combinations include IPV and Hib; IPV and MenC; IPV and MenA and C; IPV and Hib and Men C or IPV, Hib, Men A and C. Each bacterial polysaccharides may be present in doses of 1-5μg, 5-10μg, 10-20μg or 20-40μg.

Bacterial polysaccharides are unconjugated or conjugated to a carrier protein such as tetanus toxoid, tetanus toxoid fragment C, diphtheria toxoid, CRM197, pneumolysin or Protein D (US6342224).

The polysaccharide conjugate are prepared by any known coupling technique. A preferred conjugation method relies on activation of the polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is coupled directly or via a spacer group to an amino group on the carrier protein. Preferably, the cyanate ester is coupled with hexane diamine and the amino-derivatised polysaccharide is conjugated to the carrier protein using heteroligation chemistry involving the formation of the thioether linkage. Such conjugates are described in PCT published application WO93/15760 Uniformed Services University.

The conjugates are optionally prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide activated polysaccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256).

<u>Freeze drying</u>

One process of the invention involves freeze drying IPV, preferably in the presence of a bacterial polysaccharide. In this process, the preservation sample is subjected to reduced temperature and pressure conditions. The temperature is reduced to less than 0 °C, preferably less than -10 °C, -20 °C, more preferably -40 °C or -60 °C. The pressure is reduced to less than 1mbar, preferably a pressure of, or less than 0.5, 0.1, more preferably 0.05 or 0.01 mbar. The reduced temperature and pressure conditions are maintained for at least 10, 12, 16, 20, preferably 24, 36, more preferably 48 or 72 hours.

Solvent is removed until the preservation sample dries to form a solid. Throughout this application, solid includes glasses, rubbers and crystals which form as the sample

dries. Such solids retain a water content of 4-5%, 3-4%. 2-3%, preferably 1-2% or 0-1%.

Foam drying

A preferred process of the invention involves subjecting the preservation sample to such pressure and temperature conditions so that the sample begins to bubble, forming a foam.

The temperature within the preservation sample will be different from that external to the sample due to the endothermic nature of the evaporation process. References to temperature are to the conditions external to the preservation sample, for instance, where a large industrial freeze dryer is used, to the temperature of the shelf. This usually corresponds to the freeze dryer temperature setting.

A preferred embodiment of the invention achieves this by reducing the pressure while maintaining temperature conditions. The pressure is adjusted to at or below 8 , 7, 6, preferably 5, 4, 3, more preferably 2, 1.5, 1, most preferably 0.8 or 0.5 mbar, while maintaining the temperature setting at a temperature above 0 °C, preferably of between 10 °C to 15 °C; 15 °C to 20 °C; 20 °C to 25 °C; 25 °C to 30 °C; or 30 °C to 37 °C. These conditions are maintained for at least 1, 2, 3, 4, 5, 8, 10, 12, 16 or 24 hours.

Another embodiment of the invention achieves foam formation by changing the temperature while maintaining reduced the pressure conditions. The temperature setting is increased to above 20 °C, preferably to between 20 °C and 30 °C; 30 °C and 40 °C; 40 °C and 50 °C; or 50 °C and 70 °C; or the temperature setting is in the range of 10-50 °C, preferably 20-40 °C, more preferably 25-35 °C. Pressure conditions are maintained at a reduced level of or below 8 , 7, 6, preferably 5, 4, 3, more preferably 2, 1.5, 1, most preferably 0.8 or 0.5 mbar. These conditions are maintained for at least 1, 2, 3, 4, 5, 8, 10, 12, 16 or 24 hours.

Removing solvent to form a foamed glass

A subsequent stage of the foam drying method of the invention involves removing solvent until the foam dries to form a solid. In one embodiment of the invention, this is achieved by maintaining the pressure and temperature conditions at those applied in order to achieve foam formation. For instance, the pressure is maintained at or below 8 , 7, 6, preferably 5, 4, 3, more preferably 2, 1.5, 1, most preferably 0.8 or 0.5 mbar while maintaining the temperature setting at a temperature above 0 °C, preferably between 10 °C and 20 °C; 20 °C and 30 °C; 30 °C and 35 °C, most preferably between 5 °C and 25 °C. These temperature and pressure conditions are maintained for 1, 2, 3, 4, 5, 6, 8, 10, 12, 18 hours or more in order to obtain a solid with a solvent content less than or equal to 5, 4, preferably 3, 2 or most preferably 1%.

Another embodiment of the invention increases the temperature setting during solvent removal to a higher temperature setting than that maintained earlier in the process. This advantageously allows the solvent to leave the sample at a quicker rate so that the method of the invention can be completed in a shorter time. For instance, the temperature setting is increased to above 0 °C, preferably between 10 °C and 20 °C; 20 °C and 30 °C; 30 °C and 40 °C; 40 °C and 50 °C; 50 °C and 60 °C while maintaining the pressure at or below 8 , 7, 6, preferably 5, 4, 3, more preferably 2, 1.5, 1, most preferably 0.8 or 0.5 mbar. These temperature and pressure conditions are maintained for 1, 2, 3, 4, 5, 6, 8, 10, 12, 18 hours or more in order to obtain a solid with less than 5, 4, preferably 3, 2 or most preferably 1% water content.

Another embodiment of the invention reduces the pressure setting during solvent removal to a lower pressure setting than that used during foam formation. This advantageously allows the solvent to leave the sample at a quicker rate so that the method of the invention can be completed in a shorter time. For instance, the pressure setting is decreased to at or below 5, ,4, 3, preferably 2, 1, 0.8, more preferably 0.5, 0.1. most preferably 0.05 or 0.01mbar, while maintaining the temperature at or above 0 °C, preferably between 10 °C and 20 °C; 20 °C and 30 °C; 30 °C and 35 °C or

above 40 °C. These temperature and pressure conditions are maintained for 1, 2, 3, 4, 5, 6, 8, 10, 12, 18 hours or more in order to obtain a solid with a solvent content less than or equal to 5, 4, preferably 3 or 2 or more preferably 1%.

Foam drying including a freezing step

The method of the invention optionally involves freezing the sample. Freezing the sample prior to foam drying has the advantage of increased reproducibility between samples in a batch. This is due to all the samples starting the process from the same physical condition of being frozen. The preservation samples may be wholly or partially frozen.

Freezing is optionally carried out before subjected the sample to reduced pressure by placing the preservation sample at a temperature below 0 °C for a suitable amount of time to allow the sample to freeze. Preferably the temperature used is at or below -10 °C, -15 °C, -20 °C, -30 °C, -40 °C, -70 °C or -140 °C. The sample may be left at a temperature below 0 °C for 1, 2, 3, 4, 5, 8, 16 or more hours to allow freezing to occur.

For some samples, particularly samples that are easily damaged by solvent crystal formation such as cell preparations or other biological systems , it is preferable to freeze the sample slowly at a rate of less than or equal to 0.1, 0.5, 1, 2, 3, 4, 5 °C per hour. Other compositions are preserved more effectively by freezing instantaneously, for instance by snap freezing in liquid nitrogen. This method is particularly beneficial for proteins or viral particles. Freezing by evaporation also results in rapid freezing of the sample.

Alternatively, the preservation sample is frozen by subjecting the sample to reduced pressure such that the sample becomes wholly or partially frozen. Such quench freezing is carried out within a bulk freeze dryer apparatus, at a shelf temperature of or above 0 °C, 10 °C, 15 °C, 20 °C, 30 °C, 37 °C. Preferably the shelf temperature is between 5 and 35°C, more preferably between 10 and 20 °C, most preferably at

15 °C. The pressure is optionally reduced initially to 200mbar for 5, 10, 20, 30, 60 minutes or more to allow degassing. In order to freeze the sample, the pressure is reduced further to a pressure equal to or below 2, 1, 0.5, 0.2, 0.1mbar. This pressure is maintained for at least 5, 10, 20 or 30 minutes until the sample is wholly or partially frozen.

Subsequent steps of foam formation and removing solvent to form a solid are as described above.

In a preferred embodiment of the invention, the steps of freezing the sample within the freeze dryer and foam formation are performed at a constant temperature, preferably altering the pressure conditions.

In a further preferred embodiment the steps of freezing the sample within the freeze dryer, foam formation and solvent removal to form a solid, are performed at a constant temperature, preferably altering the pressure conditions.

In a further embodiment of the invention, both pressure and temperature conditions are different during the steps of freezing the sample, foam formation and solvent removal to form a solid.

The processes of the invention preferably use containers with a water repellent interior surface. This is achieved through coating the interior surface with a hydrophobic composition, for instance by siliconisation. Siliconisation is achieved by processes that are well known to those skilled in the art. Alternatively, the water repellent interior surface is achieved by the container being made of a water repellent composition.

The presence of a water repellent interior surface of the container makes foam formation more likely to occur and more reproducible. This allows lower polyol concentrations to be used in the preservation sample which in turn decreases the length of time necessary to dry the sample, reduces the effect of Maillard reactions or

other harmful interactions between the polyol and the active agent. Where the preservation samples comprises a vaccine, the resultant solid is reconstituted quickly due to the lower amount of polyol present and the resultant vaccine solution is less viscous, allowing easier administration.

All references or patent applications cited within this patent specification are incorporated by reference herein.

Examples

The examples below are carried our using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1. Evaporative freezing process

The process was carried out using a Heto Drywinner 8-85 freeze-dryer in which shelf temperature may be regulated to within 1 °C , the final temperature of the condenser is -85 °C , pressure is regulated with a bleed valve and 6 thermocouples are available to measure the product temperature.

A preservation sample was made by adding a stabilising agent (either 10% trehalose or 3.5% sucrose) and an active agent to an aqueous solution. Samples were put into the freeze dryer with a shelf temperature maintained at a fixed temperature setting of 15 °C throughout the process. The pressure was initially reduced to 200mBar and maintained at this level for 10 minutes before reducing the pressure further. At 1.5mBar, the solutions began to freeze due to evaporative cooling as shown in figure 1. The pressure is further reduced to 0.1mBar to allow the samples to become fully frozen. The pressure was then increased to between 0.8mBar and 3.5mBar at which point a foam formed as water was lost from the sample. Under the conditions of the experiment, no boiling was seen in a control sample containing only water. The samples may be loosing water through evaporation rather than through boiling. After 18 hours under these conditions, the samples are dried and the foamed solution becomes a foamed glass.

A similar process was successfully performed keeping the shelf temperature at other temperature settings up to 37 °C.

Example 2. Establishment of freezing conditions

Samples were made by dissolving sucrose in water to give 1%, 5%, 10% and 20% solutions. Samples were put into the freeze dryer with a shelf temperature maintained at 15 °C throughout the process. The pressure was initially reduced to 200mBar and maintained at this level for 10 minutes before reducing the pressure further to 50mBars, 5mBars, 2.5mBars, 0.75mBars, 0.4mBars and 0.2mBars. Each pressure level was maintained for 20 minutes to allow the temperature to equilibrate and the temperature of the sample was read using a thermocouple. Thermocouples were attached to samples with different sucrose concentrations and the temperatures recorded in table 1 are mean values of the temperatures.

Results

All samples froze between 1.66 and 1.11mbars, irrespective of the concentration of sucrose present. The temperatures measured at different pressures were very close to those predicted from the triple point curve. Therefore the presence of sucrose does not appear to have a large effect on the temperature of the samples at different pressures.

Table 1

Pressure	Measured temperature	Theoretical temperature	Liquid/frozen
1000mBar	15 °C		liquid
50mBar	15 °C		liquid
5mBar	1 °C	1 °C	liquid
2.5mBar	-5 °C	-7 °C	liquid
0.75mBar	-21 °C	-21 °C	frozen
0.4mBar	-22 °C	-27 °C	frozen
0.2mBar	-27 °C	-32 °C	frozen

Example 3. Foaming conditions for samples with different sugar concentrations

Preservation samples containing 0%, 5%, 10%, 15%, 20%, 25% and 50% sucrose were made. Samples were put into the freeze dryer with a shelf temperature maintained at 15 °C throughout the process. The pressure was initially reduced to 200mbars and maintained at this level for 10 minutes before reducing the pressure further. The pressure was further reduced to 0.1mbars to allow the samples to become fully frozen. The pressure was then increased to either 0.788mbars, 0.812mbars or 3.5mbars in subsequent experiment These conditions were maintained for 3 hours for the 3.5mbars and 0.812mbars experiments and for 6 hours for the 0.788 mbars experiment. The physical characteristics of each sample were evaluated.

<u>Results</u>

As shown in table 2, at a pressure of 3.5mbars, a high sucrose concentration of 50% was required for reliable formation of foam. In contrast, a lower pressure of 0.8mbars allowed reliable foam formation at lower sucrose concentrations of 10-25%. The use of lower sucrose concentration could be advantageous for preserved samples to be used in vaccines for instance. Therefore a process using 0.8mbars and a low sucrose content is preferred.

Table 2

Pressure	%sucrose	Physical characteristics
3.5mbars	20	4/5 foamed, 1/5 viscous liquid
3.5mbars	25	2/5 foamed, 3/5 viscous liquid
3.5mbars	50	5/5 foamed
0.812mbars	5	Ring of crystallisation and bubbles
0.812mbars	10	All foamed
0.812mbars	15	All foamed
0.812mbars	20	All foamed
0.812mbars	25	All foamed
0.788mbars	5	Ring of crystallisation and bubbles
0.788mbars	20	All foamed
0.788mbars	25	All foamed
0.788mbars	50	Foam and syrup

Example 4. The effect of using siliconized containers

Preservation samples containing 5%, 10%, 15% and 25% sucrose were made and added to vials, some of which were siliconized. In one experiment, samples were put into the freeze dryer with a shelf temperature maintained at 15 °C throughout the process. The pressure was initially reduced to 200mbars and maintained at this level for 10 minutes before reducing the pressure further. The pressure was further reduced to 2.8mbars for 3 hours. During this period, the pressure fell to 2.00mbars as the presence of water vapour decreased. The physical characteristics of each sample were evaluated.

In a second experiment, samples were put into the freeze dryer with a shelf temperature maintained at 37°C throughout the process. The pressure was initially reduced to 200mbars and maintained at this level for 10 minutes before reducing the pressure further. The pressure was further reduced to 2.4mbars for 3 hours.During this

period, the pressure fell to 1.06mbars as the presence of water vapour decreased. The physical characteristics of each sample were evaluated.

Results

Siliconization had an effect on the degassing of the samples. The reduction of pressure to 200mbars resulted in degassing of samples in siliconized vials but not in unsiliconized vials. Degassing was seen by bubbling of the sample.

The siliconisation of the vial also made foam formation more likely to occur and more reproducible (table 3). Siliconisation of vials allows foam formation to occur reproducibly at lower polyol concentrations. The lower polyol concentration decreases the length of time necessary to dry the sample and reduces the effect of Maillard reactions or other interactions with the polyol harming the active agent. Where the sample involved is a vaccine, this reduces the viscosity of the sample and allows easier administration.

Table 3

Temperature and pressure	% sucrose	Characteristics nonsiliconised vial	Characteristics siliconised vial
15°C, 2.8mbars	5%	Viscous fluid	
15°C, 2.8mbars	10%	Viscous fluid	foamed
15°C, 2.8mbars	15%	Viscous fluid	
15°C, 2.8mbars	25%	Viscous fluid	
37°C, 2.4mbars	5%	3 viscous fluid 2 foamed	
37°C, 2.4mbars	10%	All viscous fluid	5 foamed 1 viscous fluid
37°C, 2.4mbars	15%	All foamed	
37°C, 2.4mbars	25%	All foamed	

Example 5. Comparison of preservation of Hib-IPV by conventional freeze drying or by foam drying

The active agent to be preserved was a mixture of the PRP polysaccharide of Haemophilus influenzae b (Hib) and three strains of inactivated polio virus (IPV). The preservation sample was made by dissolving Hib-IPV in either a 3.15% sucrose solution or a 10% trehalose solution.

The samples were lyophilised either by using a conventional freeze drying sample that required three days to perform in a large freeze dryer, or by using the foam drying method described in example 1.

The samples were reconstituted in water and an ELISA was used to assess the integrity of structure of the three polio virus strains. Three polyclonal antibodies and three monoclonals, one against each strain, were used in separate ELISAs. Results are presented as a percentage of the reading given for a sample which had not undergone the freeze drying or foam drying procedure.

The preserved samples are assessed for their immunogenicity in vivo by inoculating groups of ten mice with the reconstituted IPV-Hib, withdrawing blood from the mice and monitoring levels of antibodies against IPV and Hib polysaccharides, for instance by ELISA or Western blotting. The degree of protection is assessed in a challenge mouse model.

Results

Using either sucrose or trehalose as the polyol, the integrity of IPV was maintained better using the foam drying technique compared to using conventional freeze drying.

Table 4

Method of drying	Polyol content	ELISA – type 1/2/3 %	
		Polyclonal	Monoclonal
Freeze drying	3.15% sucrose	46/49/58*	25/0/0
Foam drying	3.15% sucrose	85/97/106	55/68/57
Freeze drying	10% trehalose	47/43/58	
Foam drying	10% trehalose	93/86/84	72/75/87

* The experiment freeze drying in the presence of 3.15% sucrose was repeated five times and the results shown are from one representative experiment.

Example 6. Protective effect of freeze drying IPV in the presence of Hib polysaccharides.

Preservation samples were prepared containing 3.15% sucrose and IPV or a mixture of IPV and Hib polysaccharides. The samples were inserted into a Heto Drywinner 8-85 freeze-dryer and freeze dried at a temperature setting of -32 °C for 40 hours followed by continued drying at 4 °C for 16 hours.

The samples were reconstituted in water and an ELISA was used to assess the integrity of structure of the three polio virus strains. Three monoclonal antibodies, one against each strain, were used in separate ELISAs to assess the degree of antigen retention in the reconstituted, freeze dried sample compared to a reference sample that had not been frozen. Results are presented as a percentage of the reading given for a sample which had not undergone the freeze drying or foam drying procedure.

Results

As shown in table 5, the presence of Hib polysaccharide in the preservation sample with IPV, led to greater retention of IPV antigens after freeze drying than that achieved when IPV was freeze dried alone. The Hib polysaccharides have a preserving effect on IPV antigenicity in addition to that achieved by having sucrose present as a stabilising agent.

Table 5

Composition freeze dried	Polyol content	ELISA – type 1/2/3 %
IPV	3.15% sucrose	26/25/0
IPV-Hib	3.15% sucrose	52/68/0

Example 7. Effect of different stabilising agents on freeze drying IPV-Hib

Preservations samples were made containing IPV-Hib and using either 3.15% sucrose; 2.5% sorbitol, 0.8% glutamine and 0.01% HSA; MMR stabiliser and lactose; 3% glycine, 2% arginine and 4% sucrose; or 4% sucrose and 2% glycine as stabilising agent. The experiment included a sample with 3.15% sucrose as stabilising agent using double the concentration of IPV-Hib. The samples were freeze dried using a conventional three day freeze drying cycle in a batch freeze dryer.

The samples were reconstituted in water and an ELISA was used to assess the integrity of structure of the three polio virus strains. Three polyclonal antibodies and three monoclonals, one against each strain, were used in separate ELISAs. Results are presented as a percentage of the reading given for a sample which had not undergone the freeze drying or foam drying procedure.

The preserved samples are assessed for their immunogenicity in vivo by inoculating groups of ten mice with the reconstituted IPV-Hib, withdrawing blood from the mice and monitoring levels of antibodies against IPV and Hib polysaccharides, for instance by ELISA or Western blotting. The degree of protection is assessed in a challenge mouse model.

Results

Increasing the dose of IPV from 40/8/32 DU/dose to 80/16/64 DU/dose led to an increase in retention of antigenicity of IPV as shown in table 6. Variation in the stabilising agent also influenced retention of antigens with 4% sucrose/2% glycine and 2.5% sorbitol/0.8% glutamine/0.01% HAS producing higher retention of antigens as shown by ELISA data.

Table 6

Stabilising agent	Polyclonal ELISA results	Monoclonal ELISA results
3.15% sucrose	50/50/70	25/0/0
2.5% sorbitol 0.8% glutamine 0.01% HSA	55/72/72	33/50/0
MMR stabiliser lactose	59/62/65	28/25/0
3.15% sucrose Double dose of IPV-Hib	84/92/120	102/138/0

3% glycine 2% arginine 4% sucrose		
4% sucrose 2% glycine	46/62/78	25/50/15

Example 8. Reproducibility of sample quality after freeze drying, foam drying or foam drying with a freezing step.

Preservation samples are made up comprising IPV, mumps, measles, rubella, varicella zoster virus, CMV, hepatitis, HSV1, HSV2, respiratory syncitial virus, dengue, paramyxoviridae such as parainfluenza, togaviridae and influenza viruses, and/or Hib as the active agent. The active agent is dissolved in an aqueous solution containing a polyol. Multiple samples are preserved by either freeze drying, foam drying using a freezing step following the protocol described in example 1, or foam drying without a freezing step using a protocol described in example 4. Samples are reconstituted in an aqueous solution and their activity assessed. This is accomplished using ELISA assays as described in example 5 using antibodies specific to native antigens. In the case of live viruses, the titre of each sample is established by using the virus to infect suitable host cells and assessing the infectivity by plaque formation or by immunocytochemistry. Where immunogenic compositions or vaccines are foam dried, the integrity can be tested in an animal model by immunising groups of animals with vaccine which is foam dried or freeze dried and boosting the immune response for instance at 14 and 28 days after the first immunisation. Serum is isolated from animals at the end of the immunisation schedule and its titre against the vaccine is tested using standard assays, for instance by ELISA, immunocytochemistry, Western blotting, immunoprecipitation, serum bacteriocidal assay or agglutination assay. Results are complied, first by comparing the activity of the active agent after freeze drying, foam drying with a freezing step, or foam drying without a freezing step. Secondly, the degree of reproducibility of the preservation technique is assessed by

comparing the range of activities after subjecting samples to each of the three preservation methods.

Example 9. Long term storage of active agents preserved by freeze drying, and foam drying.

Preservation samples are made up comprising IPV, mumps, measles, rubella, varicella zoster virus, CMV, hepatitis, HSV1, HSV2, respiratory syncitial virus, dengue, paramyxoviridae such as parainfluenza, togaviridae and influenza viruses, and/or Hib as the active agent. The active agent is dissolved in an aqueous solution containing a polyol. Multiple samples are preserved by either freeze drying, foam drying using a freezing step following the protocol described in example 1, or foam drying without a freezing step using a protocol described in example 4. Samples are aged by storing at 37°C or 23°C for seven days and are compared for activity with samples that have been keep at 4°C . Samples are reconstituted in an aqueous solution and their activity assessed. This will be accomplished using ELISA assays as described in example 5 using antibodies specific to native antigens. In the case of live viruses, the titre of each sample is established by using the virus to infect suitable host cells and assessing the infectivity by plaque formation or by immunocytochemistry. Results are complied, first by comparing the activity of the active agent after storage at elevated temperatures with storage at 4°C. Secondly, the degree of reproducibility of the preservation technique is assessed by comparing the range of activities after subjecting samples to each set of conditions.

Claims

1. An immunogenic composition comprising IPV and a stabilising agent formulated as a dried solid, which is capable of generating an immune response against polio virus.

2. An immunogenic composition of claim 1 comprising IPV and a bacterial polysaccharide, both formulated as a dried solid, which is capable of generating an immune response against polio virus.

3. The immunogenic composition of claim 1 or 2 comprising a capsular polysaccharide or oligosaccharide antigen from *Haemophilus influenzae* b (Hib).

4. The immunogenic composition of claim 3 wherein the Hib polysaccharide or oligosaccharide is conjugated to a carrier protein.

5. The immunogenic composition of claim 4 wherein Hib polysaccharide is conjugated to tetanus toxoid.

6. The immunogenic composition of claim 2-5 wherein the Hib polysaccharide or oligosaccharide is adsorbed onto aluminium phosphate.

7. The immunogenic composition of claim 1-6 comprising a capsular polysaccharide or oligosaccharide derived from *N. meningitidis* C.

8. The immunogenic composition of claim 1-7 additionally comprising a capsular polysaccharide or oligosacchairde derived from any of *N. meningitidis* A, Y or W or combination thereof.

9. The immunogenic composition of claim 7-8 wherein meningococcal polysaccharides or oligosaccharides are conjugated to a carrier protein.

10. The immunogenic composition of claim 9 wherein Hib polysaccharide or oligosaccharide and at least one meningococcal polysaccharide or oligosaccharide are conjugated to the same carrier protein.

11. The immunogenic composition of claim 9 wherein Hib polysaccharide or oligosaccharide and at least one meningococcal polysaccharide or oligosaccharide are conjugated to different carrier proteins.

12. The immunogenic composition of claim 2-11 wherein the IPV and bacterial polysaccharide are freeze dried.

13. The immunogenic composition of claim 2-12 wherein the IPV and bacterial polysaccharide are incorporated into a foamed glass.

14. A method of making a vaccine comprising the step of reconstituting the immunogenic composition of claims 1-13 in an aqueous solution.

15. The method of claim 14 wherein the aqueous solution comprises D, T and P (acellular or whole cell) vaccine.

16. The method of claim 15 where the DTP vaccine is at least in part adjuvanted with aluminium hydroxide.

17. A kit comprising the immunogenic composition of claims 1-13 in one container and liquid DTP (acellular or whole cell) vaccine in a second container.

18. A vaccine comprising the immunogenic compositions of claims 1-13.

19. The vaccine of claim 18 which is reconstituted into an aqueous solution prior to use.

20. A container with a water repellent internal surface containing the vaccine of claim 18-19.

21. A method of preserving a composition comprising IPV and a bacterial polysaccharide comprising the steps of:
    a) preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent;
    b) subjecting the preservation sample to such temperature and pressure conditions that solvent is lost from the preservation sample ; and
    c) removing solvent until the preservation sample dries to form a solid.

22. The method of claim 21 comprising the steps of:
    a) preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent;
    b) subjecting the preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and
    c) removing solvent until the foam dries to form a solid.

23. The method of claim 22 comprising the steps of:
    a) preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent;
    b) inserting the preservation sample into a container with a water repellent interior surface;
    c) subjecting the container holding the preservation sample to such temperature and pressure conditions so that the preservation sample forms a foam;
    d) removing solvent until the foam dries to form a solid.

24. The method of claim 22-23 comprising the steps of:
    a) preparing an at least partially frozen preservation sample by suspending or dissolving IPV in a solution of a stabilising agent and freezing the mixture;

b) subjecting the at least partially frozen preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and

c) removing solvent until the foam dries to form a solid.

25. The method of claim 22-23 comprising the steps of:

a) preparing a preservation sample by suspending or dissolving IPV in a solution of a stabilising agent;

b) subjecting the preservation sample to reduced pressure such that the preservation sample becomes at least partially frozen;

c) subjecting the at least partially frozen preservation sample to such temperature and pressure conditions that the preservation sample forms a foam; and

d) removing solvent until the foam dries to form a solid.

26. The method of claim 18-22 wherein the preservation sample comprises IPV and a bacterial polysaccharide.

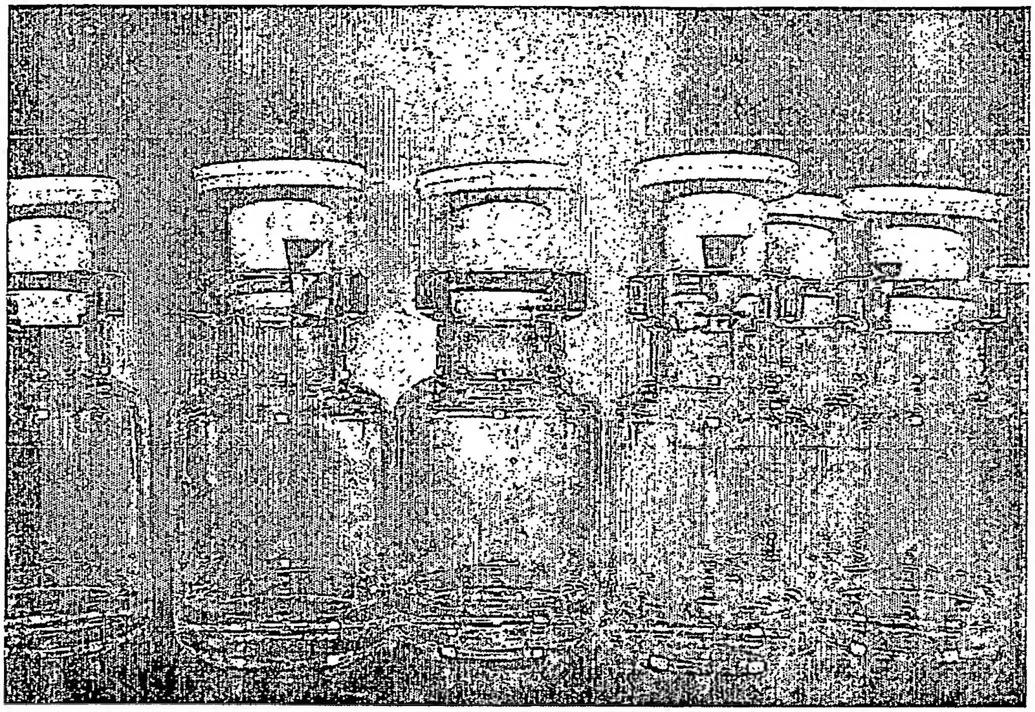
27. The method of claim 23 wherein the preservation sample comprises IPV and Hib polysaccharide.

28. The method of claim 23 wherein the preservation sample comprises IPV, and polysaccharide derived from any of *N. meningitidis* A, C, Y or W or combination thereof.

29. The method of claim 23 wherein the preservation sample comprises IPV, Hib polysaccharide and polysaccharide derived from any of *N. meningitidis* A, C, Y or W or combination thereof.

Figure 1

A



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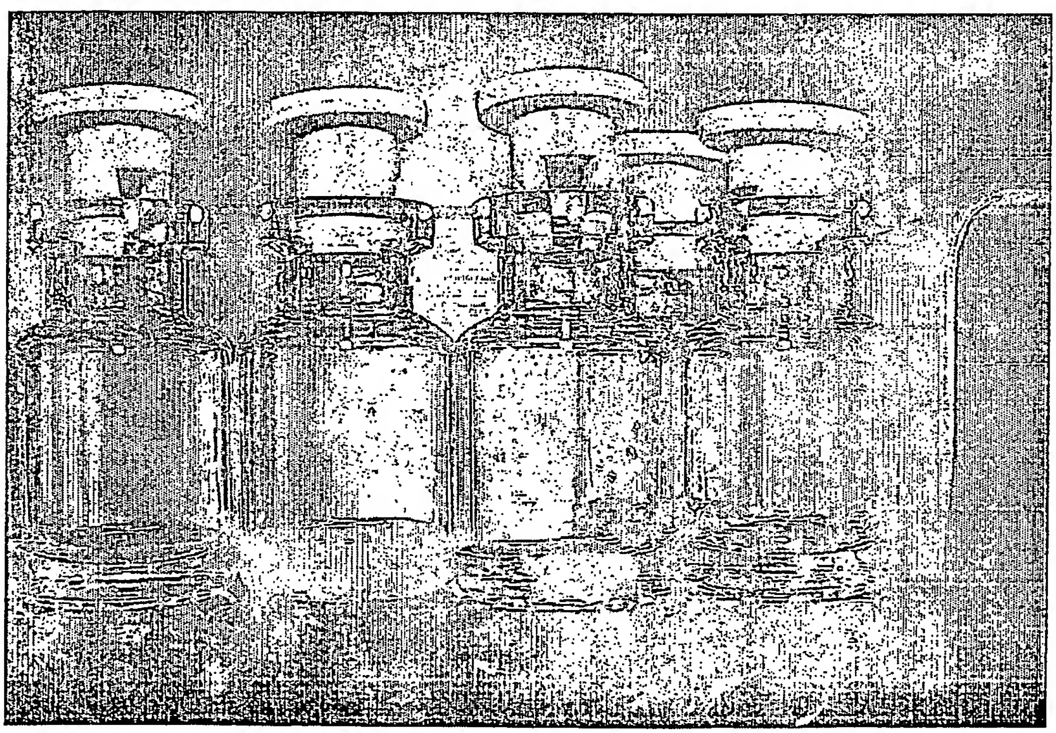

C

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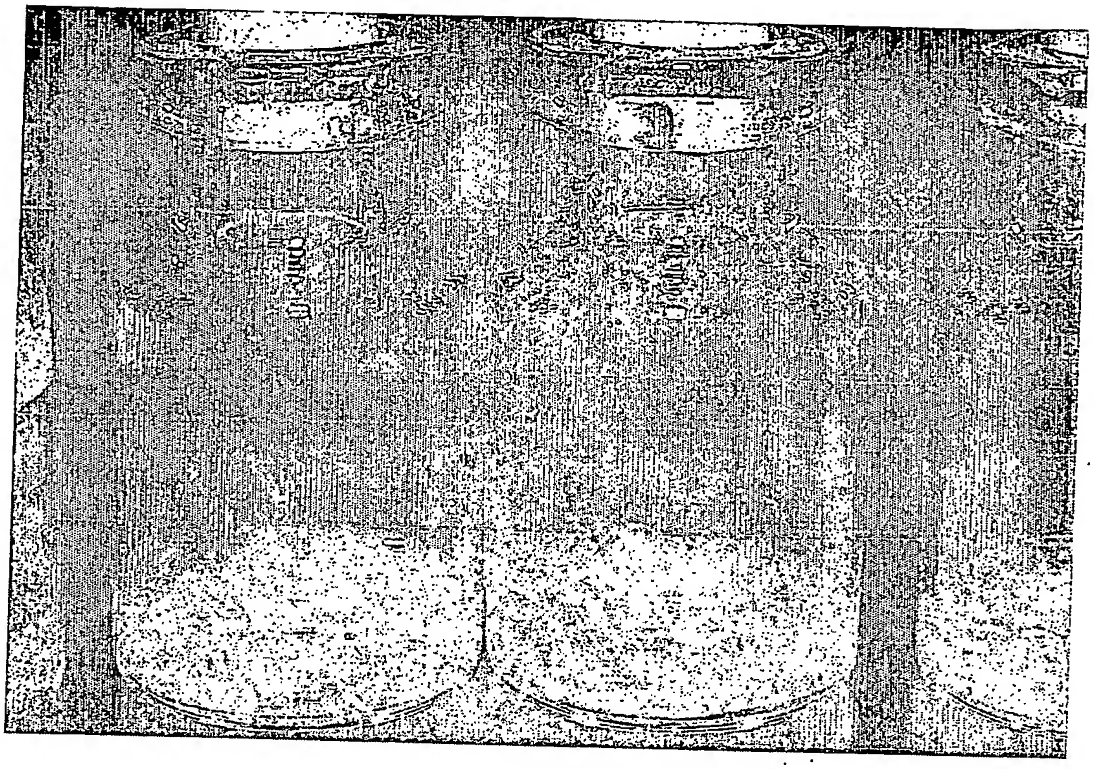
C



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